A Reversible Stoichiometric Process for Conjugating Biomolecules

Background of the Invention

Methods for reversibly linking biomolecules (e.g. nucleic acids with reporter groups or to solid supports) is important for many applications in the life sciences; it is used amongst other applications in DNA sequencing, DNA diagnostics, nucleic acid purification, Polymerase and Ligase Chain Reactions (PCR, LCR), hybridization experiments and solid phase biochemistry. Most frequently, a reversible linkage is accomplished via a streptavidin-biotin interaction (L.G. Mitchel and C.R. Merril (1989) *Anal. Biochem.*, 178, 239-242; B.H. Bowman and S.R. Palumbi (1993) in E.A. Zimmer, R.L. Cann and A.C. Wilson (ed.) *Methods of Enzymology*, Academic Press, New York, Vol. 224, pp. 399-405; X. Tong and L.M. Smith (1992) *Anal. Chem.* 64, 2672-2677).

Another reversible linkage, which is particularly amenable for linkage of nucleic acids, can be accomplished via heterobifunctional trityl groups, which can be cleaved under acidic conditions (E. Leikauf, F. Barnekow and H. Köster, Heterobifunctional Trityl Derivatives as Linking Agents for the Recovery of Nucleic Acids after Labeling and Immobilization (1995) *Tetrahedron* 51, 3793-3802; H. Köster, J.M. Coull and B. Gildea, Succinimidyl Trityl Compounds and a Process for Preparing Same, Protecting Groups for Natural Products, US Patent 5,410,068).

The interaction of metal chelates with polypeptide sequences such as oligohistidine has been used for affinity chromatography of proteins (J. Porath (1992) *Protein Express Purif.* 3, 263-281; M.C. Smith et al. (1988) *J. Biol. Chem.*, 263, 7211-7215; E. Hochuli and S. Piesecki (1992) *Methods*, 4, 68-72; E. Huchuli et al. (1988) *BioTechnology 6*, 1321-1325; E. Blum et al. (1994) *Biochem. Biophys. J.* 29, 113-121; see also European Patent No. 0 253 303 to Hoffman LaRoche AG), nucleic acids (Ch. Min and G. L. Verdine, Immobilized Metal Affinity Chromatography of DNA (1996) *Nucleic Acids Res.* 24, 3806-3810) and recently a system to detect proteins has been

introduced (Qiagen (1996): QIAexpress Detection System). Occasionally also disulfide bridges are used, which can be cleaved under reducing conditions.

However, in applications in which proteins (e.g. antibodies, enzymes) are to be linked to nucleic acids (i.e. for the detection of nucleic acids), no specific and reproducible linkage to the nucleic acids can be established, due to the fact that during chemical functionalization or activation of functional groups on the surface of the protein, no precise selection of amino acid side chains is possible and therefore neither the attachment site nor the stoichiometry can be controlled. Therefore, the results obtained can be different from batch to batch which negatively influences the generation of quantitative nucleic acid detection systems. In addition, there is no control over whether the amino acid side chain is incorporated into the active site. These factors all reduce the technical value of such procedures.

The application of solid phase techniques simplifies the preparation and purification of the reaction products, which is important for subsequent analytical and biochemical procedures. Since in some cases cleavage of one of the products from the support is needed. e.g. for further biochemical reactions in solution or signal detection, a combination of at least two different reversible linkages cleavable under mild and selective conditions is needed.

Summary of the Invention

In one aspect, the invention features compositions comprised of at least two biopolymers (e.g. nucleic acids or polypeptides), which are conjugated to an insoluble support by two different reversible linkages, which are cleavable under selective conditions.

In another aspect, the invention features novel methods and components for specifically conjugating biomolecules under completely controlled stoichiometry based on the specific and strong interaction between chelators in the presence of metal ions. In one embodiment, imidazolyl moieties are introduced via the introduction of

histidine residues (e.g. oligo-His) into a polypeptide (e.g. by recombinant DNA techniques). The oligo-His polypeptide can then interact in the presence of a metal with a nucleic acid carrying a chelator functionality at a position which is exposed and does not interfere with Watson-Crick base pairing of the nucleic acid. In another embodiment, which is particularly well-suited for the attachment of biomolecules other than polypeptides or for the reversible immobilization of nucleic acid molecules, the nucleic acid can carry a series of imidazolyl functionalities in a format which makes them available for chelation and which does not interfere with Watson-Crick base pairing; in which case, the other conjugating partner molecule can carry the chelator functionality.

By combining this reversible concept with other reversible or irreversible linkages, novel biochemical formats including diagnostic assays are possible in which favorable solid phase procedures are coupled with sensitive detection principles.

Brief Description of the Figures

Figure 1 (a) and (c) pictorially depict two general approaches of the invention in which a spacer molecule, A, linked to a polymer support, P, forms a reversible linkage, I, to a nucleic acid or protein/peptide molecule, B, which itself is linked by another reversible linkage, II, to either a nucleic acid, protein/peptide or small molecule (e.g. reporter molecule). Linkage I can be a heterobifunctional trityl group or a hydrophobic interaction stable under aqueous conditions or a photocleavable bond and II can be a bond, which is generated through a chelate complex. The two parts which form the linkage can be reversed (I', II') as shown in (b) and (d).

Figure 2 schematically depicts a nucleic acid molecule, B, which is linked through a spacer, A, via a reversible linkage, I, to a polymer support, P. B interacts via Watson-Crick complementarity with a nucleic acid molecule, C, which in turn through another reversible linkage II allows interaction with a reporter functionality D which can be a protein (enzyme), a nucleic acid or a small detector molecule.

Figure 3 schematically depicts the same approach as in Figure 2 with the

exception that B is linked to the polymer support through a spacer A with a non-reversible linkage.

Figure 4: shows an example of the chelate complex formed between a six residue histidine (his_6) tail and nitrilotriacetic acid (NTA) in the presence of Ni^{2+}.

Figure 5 schematically depicts a reaction, wherein a synthesized, protected N,N-dicarboxymethyl-serine phosphoamidite is synthesized as a chemical building block to introduce the NTA functionality into synthetic oligonucleotides.

Figure 6 shows the synthesis of a chelate-linked oligonucleotide to a his_6-BAP (bacterially generated alkaline phosphatase) conjugate by use of the phosphoamidite chelate precursor.

Figure 7 shows the synthesis of a chelate-linked oligonucleotide to his_6-BAP conjugate via retritylation and subsequent substitution with a chelate building block.

Figure 8 shows the structure of imidazolyl phosphoamidite building blocks for the single or multiple addition of an imidazolyl moiety during chemical oligonucleotide synthesis.

Figure 9 depicts the introduction of an imidazolyl moiety through an imidazolylnucleoside phosphoamidite.

Figure 10 shows the introduction of multiple imidazolyl moieties through chemical peptide synthesis of oligohistidine onto an oligonucleotide during solid phase chemical synthesis of oligonucleotides.

Figure 11 shows the chelate modified uracil and adenine nucleoside triphosphates for the enzymatic introduction of chelate functionalities into nucleic acids. Corresponding derivatives can be envisioned for cytidine, guanine or modified

nucleosides.

Figure 12 shows imidazolyl modified uracil and adenine nucleoside triphosphates for the enzymatic introduction of imidazolyl moieties into nucleic acids. Corresponding derivatives can be envisioned for cytidine, guanine and modified nucleosides.

Figure 13 schematically depicts solid phase separation/detection using NHS-DMT oligonucleotides linked to a solid phase and subsequently linked to a BAP-his_6 detector molecule via the LCR (Ligase Chain Reaction).

Figure 14 schematically depicts the detection of Polymerase Chain Reaction (PCR) products via the process of the invention.

Detailed Description of the Invention

As shown in Figure 1, two different reversible linkages I and II (a,c), which could be positioned with their functionalities reversed (I',II'; b, d) are used to link "biomolecules" or "biopolymers" (i.e.organic molecules, including nucleic acids, peptides, polypeptides) to an insoluble support. The circled P represents an insoluble or solid support.

"Insoluble supports" or ~~"soluble supports"~~ "solid supports" as used herein can be flat such as membranes, glass plates, metals, plastic films and composites thereof with a homogeneously functionalized surface or functionalized to result in an array format including flat supports with pits, wells, combs, microtiter plates, microtiter filter plates; flat supports can also be magnetic or with an array shaped (checkered) magnetic field; solid supports can also be used as beads from different plastic materials, inorganic supports such as silica, GPG (Controlled Pore Glass), metal, different polymeric material, cellulose, ~~Sephadex, Sepharose~~; the beads can be porous or non-porous, of different diameter and magnetic or non-magnetic. Also a combination of beads in the pits/wells of flat supports thus forming an array format can be employed.

Compound A can be a spacer, a nucleic acid sequence (or nucleic acid analog/mimetic) or a protein or peptide sequence, B can be a nucleic acid (or a nucleic acid analog/mimetic) or a peptide or protein, whereas C can be nucleic acid (or a nucleic acid analog/mimetic), protein/peptide or a small reporter molecule. As an example A is a spacer and I is a heterobifunctional trityl group which is coupled to a nucleic acid B; B carries a chelate functionality which interacts with the poly-his tail of a recombinant alkaline phosphatase (his_6-AP), which carries e.g. a sequence of six histidine residues at the C-terminal end of the polypeptide chain. If a chromogenic or fluorogenic substrate is added, for example, dephosphorylation generates color or light thereby providing a nucleic acid detection system. The advantage of this system is that the detection can be done either on the insoluble support or after releasing B from the support by cleavage of bond I (or I'). It is therefore possible to remove all side-products from a reaction by filtration due to the attachment to a solid phase before performing the analytical step in solution. This leads to a robust, reproducible performance.

Figure 2 shows schematically how amplification (e.g. polymerase chain reaction (PCR) or ligase chain reaction (LCR) products B-C can be captured specifically, purified and subsequently detected on the support or in solution. The first reversible linkage I (or I') e.g. a heterobifunctional trityl group anchors one strand of the LCR or PCR product via a spacer A to the support through an acid labile tritylether bond the precursor of which has been introduced by an appropriately functionalized primer during the LCR or PCR reaction. The strand C carries e.g. the chelate functionality also introduced by using an appropriately functionalized primer during PCR or LCR. The chelated moiety can then interact with a reporter functionality e.g. his_6-AP for subsequent detection and quantification of amplification product. B can also be a cDNA molecule which can be linked through its 5'-end to the polymer support. With appropriate primers, solid phase DNA sequencing can be performed. Considering an array format, this could be used for high throughput genetic and expression profiling experiments.

As shown in Figure 2, B could also be a specific (or oligo-dT) capture

sequence to select mRNA. The cDNA can be directly synthesized since the capture sequence simultaneously can act as a primer for the RNA dependent DNA polymerase. The RNA can be removed, the cDNA purified by washing and filtration steps and either released or directly used for subsequent DNA sequencing. It can also be envisioned that the capture sequence while serving as a primer for the RNA dependent DNA polymerase can be used directly to generate sequencing ladders employing ddNTP's as terminators. After purification of the sequencing ladders by washing and filtration, the bond to the polymer support is cleaved and the purified sequencing ladders subjected to either gel electrophoretic or mass spectrometric separation (H. Köster et al., A Strategy for Rapid and Efficient DNA Sequencing by Mass Spectrometry, *Nature Biotech*, (1996) 14, 1123-1128; U.S. Patent No. 5,547,835 to H. Köster; International Patent Application No. W094/21822 to H. Köster; and International Patent Application No. W096/29431 to H. Köster)

Figure 3 shows a simplified version of Figure 2 in that nucleic acid fragment B is immobilized through a non-reversible bond via a spacer A to the solid support whereas nucleic acid C carries the reporter functionality via a reversible linkage so that detection can be performed either on the support or in solution.

In Figures 1-3, biopolymer C or D could be synthetic peptides linked to an immobilized nucleic acid B or B-C respectively via a reversible linkage as described (heterobifunctional trityl, photocleavable, chelate, hydrophobic interaction) which is then detected by mass spectrometry. Various defined peptide sequences can form a specific mass tag which can be used as a specific nucleic acid identifier. Conversely specific nucleic acid sequences can be used as mass tags (specific identifiers) for proteins immobilized through a spacer A.

For use in the instant process, nucleic acids can be single stranded or double stranded polynucleotides (including oligonucleotides), whether natural or synthetic, such as deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) or DNA/RNA hybrids, DNA containing ribonucleotides and/or dideoxyribonucleotides and

RNA containing deoxyribonucleotides. Also encompassed by the term "nucleic acid" are modified nucleotides (e.g. phosphorothioate modified) as well as nucleic acid mimetics or analogs, such as peptide nucleic acids (PNAs).

As used herein, the terms "protein", "polypeptide" or "peptide" are all used interchangeably to refer to gene products. Proteins can be antibodies, enzymes, receptor molecules; peptides could be of natural or synthetic origin with oligo-his tail, a functionality for hydrophobic interaction, a photocleavable functionality or chelator functionality and displaying different properties such as being adhesive or representing specific ligand-receptor or specific protease cleavage sites.

As used herein, the term oligo his tail or poly his tail refers to a chain of conjugated histidine residues. Preferred oligo his tails contain 2-10 histidine residues. Particularly preferred oligo his tails are in the range of about 4 to about 8 his residues. Reversible linkages can be formed by hydrophobic interaction between e.g. a trityl group (i.e. with long aliphatic alkyl chains) and a long aliphatic chain e.g. attached to a polymer support or a hydrophobic polymer surface such as that of polystyrene. Since most biochemical and molecular biological reactions are performed in aqueous solution such hydrophobic interaction might be of sufficient stability. Addition of organic solvents such as alcohols, acetonitrile, N.N-dimethylformamide and the like will destabilize (if necessary in conjunction with heat) the hydrophobic interaction and release the attached molecules.

A reversible linkage which can independently be addressed could also be a functionality which is cleavable under photolytic conditions (see e.g. J. Olejnik, E. Krzymanska-Olejnik and K.J. Rothschil, Photocleavable Biotin Phosphoramidites for 5'-End-labeling, Affinity purification and Phosphorylation of Synthetic Oligonucleotides (1996) *Nucleic Acids Res.*, 24, 361-366). If the wavelength needed for photocleavage is in the range of the laser wavelength used in MALDI mass spectrometry, this bond can be cleaved during mass spectrometric signal acquisition.

A reversible linkage can also be formed from a chelator functionality which interacts with another chelator (e.g oligo-imidazolyl or other oligopeptide moieties) in the presence of a metal ion. The term "chelator" refers to a single molecule, which comprises at least two Lewis basic atoms that are capable of associating simultaneously with a Lewis acidic atom, ~~molecule~~ molecule or ion-- either simple or complex. "Lewis base" is an art recognized term that refers to chemical moieties, which are capable of donating to another atom or moiety at least one pair of unshared electrons. Examples include uncharged functional groups such as alcohols, ethers, carbonyls, thiols, sulfides, amines, imines, and pyridine and imidazole nitrogens; and charged functional groups, such as alkoxides, thiolates, carboxylates and a variety of other anions. "Lewis acid" is an art recognized term that refers to chemical moieties, which are capable of accepting from another atom or moiety (e.g. a Lewis basic atom or moiety) at least one pair of unshared electrons. Examples of Lewis acid moieties include transition metal halides, with at least one vacant d orbital, alkali metal cations, alkaline-earth metal cations, and trivalent boron or aluminum compounds. A "bidentate chelator", "tridentate chelator" and "tetradentate chelator" refers to chelators comprising two, three and four Lewis basic moieties, respectively, capable of simultaneous donation of at least an equal number of unshared electron pairs to another atom, ion or moiety.

Figure 4 depicts a specific example in which the chelator functionality is a nitrilotriacetic acid (NTA) which coordinates with divalent metal cations such as Ni^{2+} and forms a strong complex with six imidazolyl groups from a his_6 tail linked to one of the conjugating partner molecules. The term "imidazolyl residue" or "imidazoyl group" refers to any substituted or unsubstituted form of imidazole (i.e. 1,3-diaza-2,4-cyclopentadiene). For example, the side chain of the amino acid histidine comprises an imidazolyl residue.

The determination of which of the two necessary functions is attached to the nucleic acid molecule or the protein depends on the ease and convenience of introduction of either functionality (e.g. NTA or his_6 tail). In case of proteins the site-specific introduction of a chelator molecule seems to be difficult whereas the his_6 tail can

be introduced through recombinant DNA technologies. In contrast to currently available procedures, for linking nucleic acids to proteins (e.g. chemical linkage using either maleimide-thiol coupling (S.S. Gosh et al. (1990), *Bioconjugate Chem.* 1, 71-76), disulfide bonds (B.C.F. Chu L.E. Orgel (1988) *Nucleic Acids Res.* 16, 3671-3691) or mediated via streptavidin, which binds both biotinylated nucleic acids and biotynylated alkaline phosphatase (AP) (J.J. Leary et al. (1983) *Proc. Natl. Acad. Sci.* USA 80, 4045-4049)), the introduction of the his_6 tail through recombinant DNA technologies allows site-specific introduction.

As an example which does not limit the scope of this invention, the process is explained for alkaline phosphatase (AP) as protein. Alkaline phosphatase (EC 3.1.3.1) is a versatile enzyme for many molecular biological applications. It catalyzes the hydrolysis of ester bonds in phosphomonoesters and is used in recombinant DNA technology to remove 5-phosphate groups from DNA fragments to prevent self-litigation of vector DNA molecules. Coupled to antibodies or oligonucleotides, it replaces radioactively labeled compounds by serving as a reporter and signal amplifying enzyme which cleaves chromogenic or fluorogenic substrates in diagnostic applications for the specific detection of DNA (Southern blot: E.M. Southern (1975) *J. Mol Biol.* 98, 503-517) or proteins (Western blots: W.N. Burnett (1981) *Anal. Biochem.* 112, 195-203).

Predominantly, AP is isolated from calf intestine (CIP) or the bacterium E coli. (BAP). AP consists of a homodimer. The stability of the enzyme, of advantage in diagnostic applications, can lead to severe problems in cloning experiments. Residual AP activity from the dephosphorylation of vector DNA can result in dephosphorylation of the DNA to be inserted so that no or only low yields of ligation products are obtained. Heat inactivation very often is not sufficient so that time-consuming removal is necessary using treatment with proteinase K and subsequent extraction from phenol/chloroform. This lengthy procedure will also drastically reduce the yield of the product. Alteratively, AP isolated from species living at low temperatures (shrimps) are employed; here heat inactivation is possible, however, reduced stability is disadvantageous for diagnostic applications.

A modified BAP derived from E. coli was genetically designed with a his_6 tail at its carboxy terminus. The his_6 tail was introduced using inverse PCR by which six histidine codons followed by a stop codon were placed at the 3' end of the gene (E. Blum et al. (1994) *Biochem Biophys J.* 29, 113-121). To achieve high expression levels of the recombinant enzyme in E. coli, the region coding for the signal peptide of AP together with the untranslated 5' and 3' regions were exchanged with homologous sequences from the E. coli ompA gene. The expression of the resulting protein construct was under the control of the IPTG (β-D-isopropyl-thio-galactoside) inducible ptac-promoter.

The BAP-his_6 synthesized in the E.coli cell can easily be isolated from an unpurified cell extract through affinity chromatography using commercially available Ni-NTA resins (Qiagen) to which it forms a strong and specific chelate complex via its his_6 tail. The modified enzyme is therefore now available in high yields, high purity and reproducible batch-to-batch quality. As part of the inventive process, BAP-his_6 is able to form with chelate-modified nucleic acids, a stable complex which for the first time makes available specific conjugates between proteins (here BAP) and nucleic acids in a reproducible 1:1 stoichiometry.

When peptides are generated by chemical synthesis, the his_6 tail can be directly incorporated during peptide synthesis. Chemical synthesis of peptides also allows the alternative approach in which a chelator functionality is attached to the synthetic peptide either at the N- or C- terminus or one of the side chains depending on which part of the peptide sequence is needed for the biochemical function.

The nucleic acid molecule can be functionalized either with the imidazoyl moieties or with the chelator functionalities. In case of synthetic oligonucleotides the chelator functionality can be introduced in different ways. An amino acid such as serine, cysteine or lysine can be transformed into a β-cyanoethylphosphoamidite (N.D. Sinha, J. Biernat, J. McManus and H. Köster (1984) *Nucleic Acids Res.* 12, 4539-4477) carrying a precursor of the chelator functionality (e.g. NTA as described in Figure 5 and 6 with serine as starting material). During deprotection after solid phase oligonucleotide

synthesis, the three carboxyl groups are liberated forming a NTA (nitrilotriacetic acid) group linked through a phosphodiester bond to the oligonucleotide chain. In yet another way, Figure 7 shows the introduction through a heterobifunctional trityl group. The oligonucleotide is, after regular final detritylation, retritylated with a heterobifunctional trityl group bearing an active ester moiety derived from either e.g. N-hydroxysuccinimide or employing active esters such as p-nitrophenyl esters. The active ester functionality is then reacted with a chelator molecule derived from e.g. lysine.

The imidazolyl functionality can be introduced during oligonucleotide synthesis employing an appropriate β-cyanoethylphosphoamidite as shown in Figure 8; single or multiple imidazolyl residues can be incorporated. A imidazolylnucleoside as shown in Figure 9 or a histidine peptide sequence covalently attached to the oligonucleotide chain (Figure 10) can also be used to introduce the necessary imidazolyl moieties for interaction with the chelator functionality.

The chelator and oligoimidazolyl functionalities can also be introduced in high molecular weight nucleic acids using either DNA dependent DNA or RNA polymerases or RNA dependent DNA polymerases using appropriately modified nucleoside triphosphates (either NTPs, 2'-dNTP, 3'-dNTPs, ddNTPs) as depicted in Figure 11. The base will carry either the chelator or the oligoimidazoyl functionality (Figure 12) in case of pyrimidine bases at C5 and in case of purine bases at C8 so that Watson-Crick base pairing is possible. Using the appropriate nucleoside triphosphates those functionalities can either be introduced internally (NTP for RNA synthesis or 2'-dNTP for DNa synthesis) or at the 3'-end (3'-dNTP for RNA synthesis, ddNTP for DNA synthesis). The incorporation can be performed during amplification procedures such as PCR, SDA or during DNA sequencing. Those skilled in the art will realize other approaches to introduce either chelator or oligo-imidazolyl moieties into nucleic acids.

Detection of the immobilized nucleic acid-protein/peptide conjugates can be achieved either directly on the polymer support or after selective cleavage of either reversible bond I (I') or II (II'). The signal can be detected by any of a number of means

including radioactivity, fluorescence, chemiluminescence (using e.g. 1,2-dioxetan derivatives) or colorimetric (using e.g. BCIP/NBT) methods depending on the substrates used as C or D Fig. 1, 2 and 3). D can be an enzyme such as AP which triggers upon contact with a substrate through its enzymatic activity the signal generation. C and D can also be detected through their molecular weight by employing mass spectrometric methods. Preferred mass spectrometer formats for use in analyzing the translation products include ionization (I) techniques, including but not limited to matrix assisted laser desorption (MALDI), continuous or pulsed electrospray (ESI) and related methods (e.g. Ionspray or Thermospray), or massive cluster impact (MCI); these ion sources can be matched with detection formats including linear or non-linear reflectron time-of-flight (TOF), single or multiple quadrupole, single or multiple magnetic sector, Fourier Transform ion cyclotron resonance (FTICR), ion trap, and combinations thereof (e.g., ion-trap/time-of-flight). For ionization, numerous matrix/wavelength combinations (MALDI) or solvent combinations (ESI) can be employed. Subattomole leels of protein have been detected, for example, using ESI (Valaskovic, G.A. et al., (1996) Science 273: 1199-1202) or MALDI (Li, L. et al., (1996) J. Am. Chem. Soc. 118:1662-1663) mass spectrometry.

The process of the invention is further demonstrated by solid phase separation and detection of Ligase Chain Reaction (LCR) products as seen in Figure 13 and products of PCR reactions (Figure 14). To those skilled in the art it is obvious that all applications and variations of amplification procedures including those useful for the detection of mutations and DNA/RNA sequencing are all adaptable to the process of the invention thereby significantly improving such processes.

The present invention is further illustrated by the following Examples, which are intended merely to further illustrate and should not be construed as limiting. The entire contents of all cited references (including literature references, issued patents, published patent applications and co-pending patent applications, as cited throughout this application) are hereby expressly incorporated by reference.

Example 1 BAP-his_6 Fusion Protein

The phoA gene coding for the BAP of E. coli (P.E. Berg (1981) *J. Bacteriol.* 146, 660-667; C.N. Chang et al. (1986) *Gene* 44, 121-125) was derived from E. coli strain HB101. The his_6 fusion at the carboxyterminus was generated via inverse PCR with six his codons followed by a stop codon derived from plasmid pHis 1 (E. Blum et al. (1994) *Biochem. Biophys. J.* 29, 113-121).

To increase the expression rate of the recombinant BAP-his_6 protein, its reading frame was embedded in the untranslated regions of the E. coli ompA gene (Chen et al. (1991) *J. Bacteriol.* 173, 4578-4586), coding for protein OmpA, which is a major protein constituent of the outer membrane in Gram-negative bacteria. In addition, the signal peptide of BAP (H. Inouye and J. Beckwith (1977) *Proc. Natl. Acad. Sci. USA* 74, 1440-1444) and the first two amino acids of the mature protein were replaced by the OmpA leader peptide and the first amino acid residue of mature OmpA, resulting in a mature chimeric BAP with the amino acid alanine instead of arginine-threonine at its N-terminus.

To bring the expression of the chimeric BAP-his_6 under the control of IPTG inducible chimeric tac-promoter (T. Amann et al. (1983) *Gene* 25, 167-178), a 2.5 kb *EcoRI-PstI* fragment containing the complete open reading frame of the ompA-phoA chimera) and the untranslated regions from the ompA gene was cloned into the expression vector pHK236 (a derivative of pJF118u: Fürste et al. (1986), kindly provided by M. Kröger, Giessen) to generate the BAP-his_6 expression plasmid vector pBAPHIS8. Expression is achieved by induction of logarithmic E. coli culture harboring plasmid pBAPHIS8 with IPTG in a final concentration of 1 mM for 2 h under shaking in a 37°C incubator. Isolation of BAP-his_6 is carried out according to developed protocols on Ni-NTA-Agarose (E. Hochuli et al. (1987) *J. Chromatography* 411, 177-184).

Example 2 Dephosphorylation of DNA Fragments with Solid Phase Bound BAP-his_6

A solution containing DNA fragments is incubated with beads carrying immobilized metal ions complexed with BAP-his_6 protein. To remove the enzymatic activity after the reaction is carried out, filtration or centrifugation removes beads with adsorbed enzyme. Alternatively, a solution containing DNA fragment can be filtered through a derivatized membrane, carrying immobilized metal ions complexed with BAP-his_6 protein.

Example 3 Detection of LCR Products in Microtiter Filter Plates

The use of BAP-his_6 as a reporter enzyme for LCR is carried out in the wells (96 or more) of a microtiter filter plate (MTFP) with 96 samples with oligos A-D (Figure 13). One of the oligos (oligo A being the marker oligo, Fig. 13) carries at its 5'-end a chelating group. In the presence of a template DNA the marker oligo is incorporated into one strand, the marker strand, consisting of oligos A and B, with B ligated to the 3'-end of oligo A. Under denaturing conditions (or after denaturing), ligation products, oligos and other smaller by-products are transferred by suction into a second MTFP with a derivatized filter membrane. To this filter, oligo D or part of it with sequence complementary to oligo B is coupled via NHS-DMT (heterobifunctional trityl derivative) linkage. Hybridization occurs between membrane bound oligo D and oligo B or the marker strand AB. After removal of supernatant and washing, only oligo A incorporated in the marker strand AB by ligation remains in the wells of the MTFP. BAP-his_6 and a divalent cation such as Ni^{2+} are incubated in the wells under adequate conditions to allow coupling of BAP-his_6 to the marker strand. After removal of unbound BAP-his_6 by washing and filtration, chromogenic or fluorescent AP substrates are added. Only wells containing the LCR product show AP activity as a positive result, bound D alone or the single strand CD cannot give rise to any signal. The experimental setup allows multiplex LCR by employing a mixture of oligos in the LCR and subsequent transfer of the LCR products by suction through a stack of different MTFP with specific bound oligo sequences. This experiment setup is amenable to automation, since the

reaction can be carried out e.g. in filter tubes or filter plates, which allow removal of contaminating agents, buffer changes and even detection *in situ* by dispensing and filtration of different liquids.

Example 4 Sequence Specific Detection of PCR Fragments

PCR is carried out in crude cell lysates with a derivatized oligonucleotide primer (Figure 14). After denaturing, the PCR reaction is filtrated through a membrane derivatized with a capture oligo. It can contain any sequence, which is complementary to the expected PCR fragment and hybridizes with strand elongated from derivatized oligo. Although any nucleic acid containing the sequence complementary to the capture oligo will be retained on the membrane, only PCR products containing the derivatized oligonucleotide primer can bind the modified BAP-his_6 enzyme. The PCR product is detected by BAP activity retained on the membrane after adequate washing procedure. This setup allows PCR with crude lysates, since contaminating agents can be removed by filtration and only the PCR products retained by hybridization to the membrane bound oligonucleotide give rise to a detectable signal. This setup is also amenable to multiplexing (see above).

Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of the invention and are covered by the following claims.